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[COMMUNICATION]

Possible Involvement of GABAergic Neurons in Regulation of Diapause Hormone Secretion in the Silkworm, *Bombyx mori*

Isamu Shimizu, Tomohiko Matsui and Kou Hasegawa

Research Section of Environmental Biology, Laboratory for Plant Ecological Studies, Faculty of Science, Kyoto University, Kyoto 606, Japan

ABSTRACT—Injection of picrotoxin and bicuculline, blocking agents of GABA-mediated synaptic inhibition, into the silkworm pupae of non-diapause eggs producer induced diapause eggs. On the other hand injection of γ-aminobutyricacid (GABA) into diapause producers caused a production of non-diapause eggs. These two lines of evidence strongly suggest that GABAergic neurons are involved in the regulation of the diapause hormone secretion from the subesophageal ganglion of the silkworm.

INTRODUCTION

Insects enter diapause at various life stages characteristic of each species, which is governed by endocrine events [1, 2]. The silkworm, *Bombyx mori* is a typical insect species of embryonic diapause and the nature of the diapause has been well documented [3]. The embryonic diapause is determined by various environmental conditions which the mother moths have experienced during their embryonic and larval stages [4, 5]. The diapause hormone is secreted from neurosecretory cells located in the subesophageal ganglion (SG) of the diapause eggs producer during the pupal stage, acting on the developing ovaries and produces diapause eggs. In non-diapause eggs producer the secretion is blocked and the eggs laid become non-diapause [6–8]. Some pieces of evidence obtained by surgical experiments suggested that brain (Br) of the silkworm controls the secretion of the diapause hormone from SG through subesophageal connective:in the pupa of diapause producer a stimulatory signal from Br brings about the release of the diapause hormone from SG and in non-diapause producer a repressive signal suppresses it [9–11]. However, the physiological mechanism controlling the secretion has not been revealed yet.

GABA functions as an inhibitory neurotransmitter in both vertebrate and invertebrate nervous systems and GABAergic neurons usually inhibit their targets by releasing the chemical transmitter (GABA) that opens chloride channels in the post-synaptic cells [12]. Several lines of evidence demonstrated that the inhibitory neurons play an important role in integrating overall level of neural activities. Picrotoxin and bicuculline are known to block selectively and reversibly the inhibitory synapses mediated by GABA [13]. These chemical agents are used often to probe the mechanism of the GABA-mediated synaptic inhibition [14]. Using these agents we obatined experimental results suggesting that GABAergic neurons are involved in the regulation of the diapause hormone secretion in the silkworm.

MATERIALS AND METHODS

The silkworm employed was a bivoltine race (*Daizo*), whose characteristics of the photo-

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periodic response have been well documented elsewhere [15]. Two batches of female silkworms were destined to produce diapause eggs or non-diapause eggs by controlling the environmental conditions during their embryonic and larval stages [15]. Picrotoxin (Sigma) and GABA (Nakarai Chem. Ltd.) were dissolved in saline at the concentration of 30 μg/10 μl and 10 mg/10 μl, respectively. Bicuculline (Sigma) was dissolved in ethylacetate at the concentration of 160 μg/10 μl. After ether anaesthesia injections of these test solutions into the abdominal segement of pupae were carried out using microsyringes. Injection of the agents did not affect the development of adults and ovaries.

Diapause eggs develop ommochrome pigments in serosa after oviposition. Usually the appearance of this pigment and an arrest of embryonic development are used as criterions for the embryonic diapause of the silkworm. Moths injected with picrotoxin or bicuculline laid only few eggs of their ovaries, and so we used following method to detect 3-hydroxykynurenine accumulated in eggs of ovary. After adult emergence ovaries were dissected out in saline and one of eight ovaioles was cutted out from the anterior to posterior end, putted on filter paper straightly in linear order, and all eggs were crushed completely without putting out of order. Then Ehrlich's diazo reagent was dropped upon the part of the filter paper into which the egg-extracts infiltrated and red coloration indicating the presence of 3-hydroxykynurenine was examined.

3-Hydroxykynurenine is a metabolic precursor of ommochrome pigments and the diapause hormone was reported to accelerate the accumulation of the amino acid in pupal ovaries [16]. Therefore the detection of 3-hydroxykynurenine accumulated in eggs using Ehrlich's diazo reaction has been carried out to see the commitment of the hormone to the developing eggs [17, 18]. We confirmed that eggs laid by diapause producers were positive (bright-red colour) to the reaction (the criterion was $+++$ or $++$) and that eggs by non-diapause producers were slightly positive or negative ($+$ or $-$). The number and arrangement of diapause and non-diapause eggs in each ovariole were recorded.

RESULTS AND DISCUSSION

To see an involvement of GABAergic neurons in the diapause hormone secretion 90 μg of picrotoxin or 32 μg of bicuculline was injected into 4 day-old pupae of the non-diapause eggs producer (Table 1). Injection of these compounds caused continuous convulsions of pupal abdomen for 24–30 hr after the treatment, but scarcely affected the pupal development and almost all adult moths emerged 9–10 days after larval-pupal ecdysis. After adult ecdysis the diapause and non-diapause of eggs were examined by Ehrich's diazo reaction according to the criteria as described above.

In non-injected control and saline injected control, all female moths had only non-diapause eggs. On the other hand, all animals injected with

Table 1. Production of diapause eggs in non-diapause eggs producers of the silkworm pupae by injection of picrotoxin or bicuculline

Injection	ND ($-$, $+$)	Mixed ($-\sim+++$)	D ($++$, $+++$)	D% ($++$, $+++$/Total)
Control	20	0	0	0%
Saline (30 μl)	13	0	0	0%
Picrotoxin (90 μg)	0	13	0	53%
Ethylacetate (2 μl)	11	0	0	0%
Bicuculline (32 μg)	1	7	0	23%
GABA (10 mg)	12	0	0	0%

ND, Mixed and D represent the silkworm moths producing 100% non-diapause eggs, mixed eggs and 100% diapause eggs, respectively. 4 day-old pupae were used. Criterion ($-\sim+++$) represents the Ehrilich's diazo reaction. See the text in detail.

picrotoxin produced mixed batches of diapause and non-diapause eggs: the diapause percentage (eggs of +++ and ++ per total eggs examined) was 53%. There was no difference in the arrangement of diapause and non-diapause eggs in eight ovarioles of one moth. Dose response curve showed that injection of 15 μg of picrotoxin was not effective dose to produce diapause eggs, but 30 μg of the agent was effective (29% diapause). In every adult eggs of late (anterior) and middle part in each ovariole were positive to Ehrich's reaction. On the other hand, injections into 0–3 day-old pupae produced the diapause eggs in the early (posterior) group. Bicuculline (32 μg) of an antagonist of GABAergic neuron produced diapause eggs also, while ethylacetate (2 μl) of the solvent did not affect the non-diapause production at all.

After surgical removal of SG from the pupae of non-diapause producer on the day of larval-pupal ecdysis picrotoxin was injected into the operated pupae of 4 day-old. However the injection of picrotoxin produced little diapause eggs. This showed that picrotoxin did not act directly on the ovary to make eggs diapause or not increase the content of 3-hydroxykynurenine in developing ovary. It is most likely that these agents act on the central nervous system of Br-SG. Injection of GABA into non-diapause eggs producers did not affect the non-diapause production.

From the observations obtained above we tentatively assumed that GABAergic inhibitory synapses function in the repression of the diapause hormone release from SG in non-diapause producers. Then GABA was injected into the pupae of diapause eggs producers in expectation of non-diapause eggs production (Table 2). Non-injected controls and saline-injected controls of 3 day-old pupae did not produce any non-diapause egg. On the other hand injections of GABA (10 mg) into 20 pupae produced 12 moths of 100% diapause eggs and 8 moths of mixed type of diapause and non-diapause eggs. In this case the early and middle part of the ovariole became non-diapause. As the day of injection became late from 2 day to 5 day, non-diapause eggs zone of the animal in which it was effective went backward from early to late group of eggs. GABA injected was presumably effective as the synaptic inhibitor for about 24 hr after an injection before its degradation or absorption: production of non-diapause eggs by GABA may be due to a temporal repression of diapause hormone release through an inhibition of the neurosecretary cells. One mg of GABA was not effective dose, and injection of picrotoxin into diapause producers did not affect the diapause production at all.

It was suggested that series of functional components involved in the silkworm photoperiodic response, that is photoperiodic photoreceptor, clock and counter system, all reside in brain and that the hormonal-effector system is located in SG [11]. Fukuda and Takeuchi [7] concluded according to their detail histological studies that the diapause hormone was produced by a pair of neurosecretory cells located toward the ventral side of SG. They observed that the neurosecretory cells actively accumulated neurosecretory materials in non-diapause producers and released them in diapause producers. Our results obtained here suggested that the brain of the silkworm regulates the secretion of the diapause hormone from SG by innervation through GABAergic neurons and that the inhibitory regulation by the neurons is primary in the control of the hormone secretion. However

Table 2. Production of non-diapause eggs in diapause eggs producer of the silkworm pupae by injection of GABA

Injection	ND (−, +)	Mixed (−~+++)	D (++, +++)	ND% (−, +/Total)
Control	0	0	16	0%
Saline (10 μl)**	0	0	15	0%
Picrotoxin (90 μg)*	0	0	16	0%
GABA (10 mg)**	0	8	12	15%

Injections were carried out using 3 day-old (*) and 4 day-old (**) pupae, respectively.

the problem whether the GABAergic neurons project to the neurosecretory cells directly or not remains to be open. In vertebrates GABAergic neurons have been reported to function in the neuroendocrine control of hormones [19]. This is the first report suggesting an involvement of GABAergic neurons in the endocrine control of insect hormones.

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